

Methods for Perfusing the Male Reproductive Tract: Models for Studying Drug and Hormone Metabolism

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Methods for perfusing rat testis and accessory sex organs *in situ* by recirculating artificial medium to the hemicorpus preparation are described. The advantages and limitations of this system for studying the male reproductive tract were examined. The preparation was then used to study the uptake of androgens into the nuclei of caput epididymis, ventral prostate, seminal vesicle and testis. The accumulation of dihydrotestosterone and accessory sex organ nuclei was saturable and inhibited by perfusion of excess testosterone or cyproterone acetate. By contrast, testosterone was the major nuclear androgen in the testis of mature hypophysectomized preparations perfused with testosterone. In all parts of the reproductive tract, ^3H -nuclear androgens were associated with 3S, salt-extractable macromolecules within the properties of androgen receptors.

The hemicorpus preparation was extensively compared with two techniques (selective and isolated) for perfusing the testis directly. Of these two procedures, the isolated method was superior when only the testes were studied. However, the hemicorpus preparation offers the advantage of studying testes along with the remainder of the male reproductive tract. A variety of observations suggest that these perfusion procedures will be useful for the study of drug as well as hormone metabolism and mechanism of action.

Investigations of drug and hormone metabolism and mechanism of action are facilitated by the use of perfusion techniques. Perfusion offers several advantages over methods employing dissected tissue or intact animals including: control of medium composition entering the organ, delivery of substrates via the vascular bed, metabolism of drug or hormone only by the organ under study and quantitative analysis of hormone or drug transfer between functional compartments. Studies from this laboratory have characterized a method for *in situ* perfusion of the male rat reproductive tract using a hemicorpus preparation (1, 2). In addition, a technique for direct perfusion of isolated testis was de-

scribed (3). Both of these procedures are suitable for studies of LH action on testis, testosterone secretion and hormone or drug metabolism (1-3). In addition, recent experiments have indicated that these perfusion methods may be used to study the early steps of androgen action. Testosterone and its 5α -metabolites are believed to initiate their action by binding to receptors which are transferred to the nucleus of the cell. Perfusion of the male reproductive tract permits a direct comparison of androgens accumulated in nuclei of seminal vesicle, prostate, caput epididymis and testis. Perfusion studies also facilitate an examination of the relationship between intervascular concentration of testosterone and nuclear androgen levels (4).

The present report reviews the use of perfused hemicorpus and isolated testis. Representative experiments are presented to demonstrate the usefulness of these techniques for studies of androgen production and binding.

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Techniques of Perfusion

Methods of Perfusion

The perfusion apparatus described in detail by Jefferson (5, 6) and constructed by Vanderbilt University Apparatus Shop, consists of a Plexiglas box maintained at 37°C with a thermostat and lamps. Inside the box, there are duplicate drum oxygenators, preparation trays, bubble traps and connecting plastic tubing. Perfusate is circulated with Harvard peristaltic pumps (Model 1201) situated outside the box. A gas mixture (95% O₂, 5% CO₂) is delivered into the revolving drum oxygenators after humidification. Oxygenated perfusate is pumped through a wire mesh filter and passes through a bubble trap to a Teflon cannula (ID 1.48 mm, OD 2.08 mm) placed in the descending aorta of the hemicorpus preparation. The venous effluent drips from the cut inferior vena cava through a wire mesh screen to a collecting tray and is pumped back into the oxygenation drum. A T-tube in the return line permits collection of samples of the venous perfusate.

In the experiments reported here, the perfusate consisted of Krebs-Henseleit bicarbonate buffer (7) modified by an increase in calcium concentration to 3.0mM and the addition of 0.5mM disodium ethylenediaminetetraacetic acid, 17mM glucose, 30 g/l. bovine serum albumin (Pentex, Cohn fraction V), and 28% washed bovine erythrocytes. This medium was freshly prepared for each perfusion and was placed in each oxygenation drum and allowed to equilibrate with the gas mixture before the study was commenced.

Hemicorpus Technique

The rat was heparinized with an intraperitoneal injection of sodium heparin, 100 mg/kg, 30 min before anesthesia was induced with intraperitoneal sodium pentobarbital, 50 mg/kg. The abdomen was opened with a semicircular incision below the costal margins and the coeliac and superior mesenteric arteries, renal vessels, and inferior mesenteric vessels together with the rectum were ligated. Care was taken to leave the internal spermatic vessels undisturbed. The chest was opened by cutting through the left dorsal costal angles and around the costal insertion of the diaphragm. A ligature was passed around the aorta above the diaphragm and tied loosely. Blood flow was stopped by a second ligature below the arch of the aorta. A small hole was cut in the aorta approximately 1 cm above the diaphragm and the Teflon cannula inserted so that its tip was above the origins of the renal arteries. The

cannula was then secured by tightening the lower ligature. Perfusion at a flow rate of 7 ml/min was started immediately after placement of the cannula. The time from opening the chest to commencement of perfusion was less than 90 sec. The upper half of the animal was transsected above the cannula, the intestine, liver, and adrenal glands were removed, and the inferior vena cava was cut at the level of the right crus of the diaphragm. The abdominal cavity was flushed with physiological saline and covered with a plastic sheet. The rate of perfusion was then increased to 35 ml/min. The first 50 ml of perfusate which flowed out the T-tube in the venous line was discarded. The T-tube was closed and perfusate permitted to recirculate in some studies and remained open for a single flow-through or nonrecirculating procedure in others (2).

Isolated Perfusion Technique

Rats were anesthetized with sodium pentobarbital, and testes were exposed through a scrotal incision. A ligature was placed around the straight dorsal capsular portion of the internal spermatic artery, taking care not to puncture the adjacent veins or include the underlying parenchymal portion of the internal spermatic artery. A 27 gauge needle on the end of the perfusion tubing was inserted into the artery and secured with the ligature. Perfusion was started immediately. The testis was then dissected away from the epididymis and placed in a plastic funnel. The perfusion apparatus was modified for the simultaneous perfusion of eight testes at 33°C. Gilson roller pumps were used to perfuse the testes at 0.35 ml/min and the venous effluent was collected in beakers (3).

Characterization of the Perfusion Techniques

Characterization of Hemicorpus

A thorough evaluation of this perfusion system was reported in detail previously (1, 2). The electrolytes, pH and gas composition of the perfusate remained constant for up to 3 hr of perfusion, and the glucose levels, while falling, still remained in the normal range. Other characteristics of this system are summarized in Figure 1. It is significant that even when most of the reproductive tract was hyperperfused, blood flow to the testis still remained below that of an intact animal (2). Although testicular ATP and GTP levels were normal after 90 min of perfusion, these values fell substantially by 180 min.

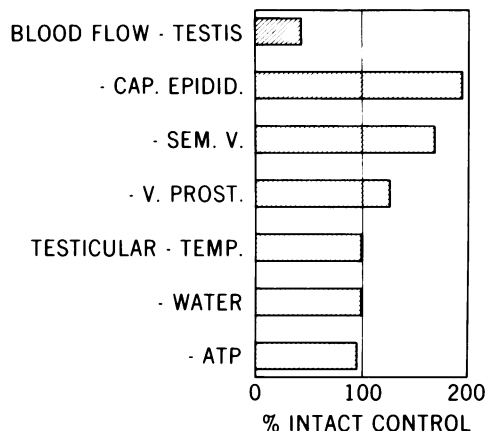


FIGURE 1. Properties of the hemicorpus preparation perfused at 35 ml/min for 90 min.

Characterization of Isolated Testicular Perfusion Technique

With this method, testicular water content, temperature and flow rates were normal (Table 1); however, ATP and GTP levels were low after 3 hr of perfusion (3), as was the case with the hemicorpus technique (2). Many attempts were made to prevent this decline in high energy phosphate but without success.

Both of the above methods can be used for studies of testicular function. However, if only the testis is to be studied, the isolated perfusion technique has several advantages over the hemicorpus preparation. Isolated testes require less medium and maintain normal flow rates and water content for prolonged periods. Another advantage is that there is virtually no ischemic period at the commencement of perfusion. Furthermore, as noted below, the response to hCG was more sensitive and testosterone secretion less variable. These latter re-

Table 1. Comparison of testes from intact rats with those perfused by the isolated technique.^a

	Intact	Perfused 3 hr
Water content, ml/g	0.875 ± 0.002 (18)	0.876 ± 0.006 (11)
Temperature, °C	32.7 ± 0.6 (12)	32.7 ± 0.4 (14)
ATP, μmole/g	2.50 ± 0.4 (12)	0.60 ± 0.4 ^b (12)
GTP, μmole/g	0.27 ± 0.04 (12)	0.07 ± 0.05 ^b (12)
Blood flow, ml/g-min	0.221 ± 0.028 (12)	0.214 ± 0.029 (19)

^a All values are means ± SD; number of measurements in parentheses.

^b Significantly different from value for intact rats ($p < 0.01$).

sults were probably a consequence of constant flow to the isolated preparations in contrast to the smaller flow through testes of the hemicorpus.

Secretion of Testosterone

Hemicorpus Preparation

Without tropic hormone in the perfusate, a low and constant testosterone concentration was achieved after 30 min. The addition of hCG increased testosterone secretion and resulted in a plateau after 90 min. The dose-response relationship of hCG stimulated testosterone secretion is shown in Figure 2. The testosterone levels were measured in samples taken at 90 min of perfusion with medium containing different concentrations of hCG. There was a logarithmic dose response over the range of 50–1000 mIU/ml of hCG. At a higher concentration of hCG, there was inhibition of testosterone secretion (2).

Selective Testicular Perfusion

As with the hemicorpus method, a maximal stimulatory dose of hCG produced an increase in testosterone secretion over a 1 hr period of time. There was also a dose-response relationship between hCG in the medium and the testosterone secretion rates. In this instance, testosterone secretion was maximal at 100–500 mIU/ml and was reduced by 1000 mIU/ml (3).

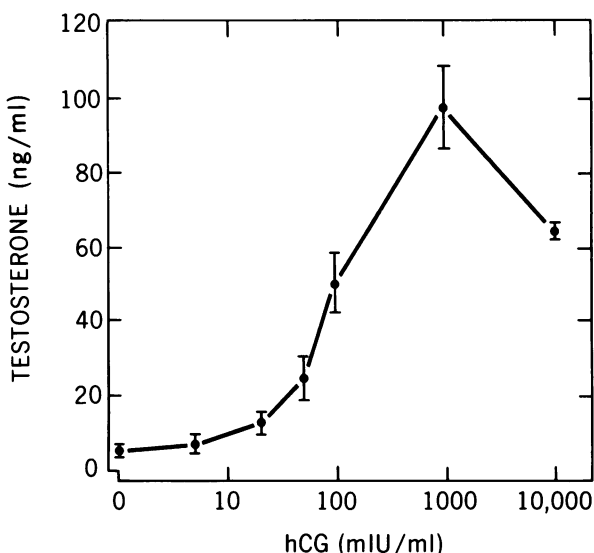


FIGURE 2. Dose-response relationship between hCG and testosterone concentration at 90 min. Mean ± SEM for 5-12 samples. From Baker (2).

These studies emphasize that both the hemicorpus and isolated testicular techniques can be used for studies of testosterone secretion. As noted above, the isolated testis technique is more sensitive and less subject to variability (3).

Nuclear Accumulation of Androgens in the Perfused Reproductive Tract

Methods

For studies of androgen receptor binding, rat hemicorpora were perfused for 60 min with ^3H -testosterone of various specific activities (0.56–59 Ci/mmol) using a recirculating system. In some experiments, isolated testes were perfused with testosterone or dihydrotestosterone in a nonrecirculating system for 1 hr. When dihydrotestosterone was used, bovine erythrocytes were omitted from the perfusion medium to avoid steroid metabolism (4).

Following the perfusion, nuclei were isolated as previously described (8). For determination of total steroid content, nuclei were extracted with ethanol and the extracts were fractionated on thin layer chromatography (4). In some instances, nuclei from accessory sex organs and testes were extracted with two volumes of 0.4M KCl. This extract was then fractionated on 5–20% sucrose gradients (9).

Apparent Saturation of Nuclear Binding Sites

Following infusion of ^3H -testosterone, dihydrotestosterone comprised 80–90% of the steroid isolated from prostatic, seminal vesicle, and epididymal nuclei. The relationships between dihydrotestosterone levels in accessory sex organ nuclei and the initial testosterone concentration in the perfusion medium are shown in Figure 3. In each organ, nuclear dihydrotestosterone levels rose with increasing concentrations of testosterone in the medium up to 30–50 nM but thereafter remained constant. This observation suggests that the nuclear accumulation of dihydrotestosterone is a saturable process. The results in Figure 3 also indicate that nuclei of the accessory sex organs can be saturated with dihydrotestosterone when the extracellular testosterone level is near the physiological range for the male rat.

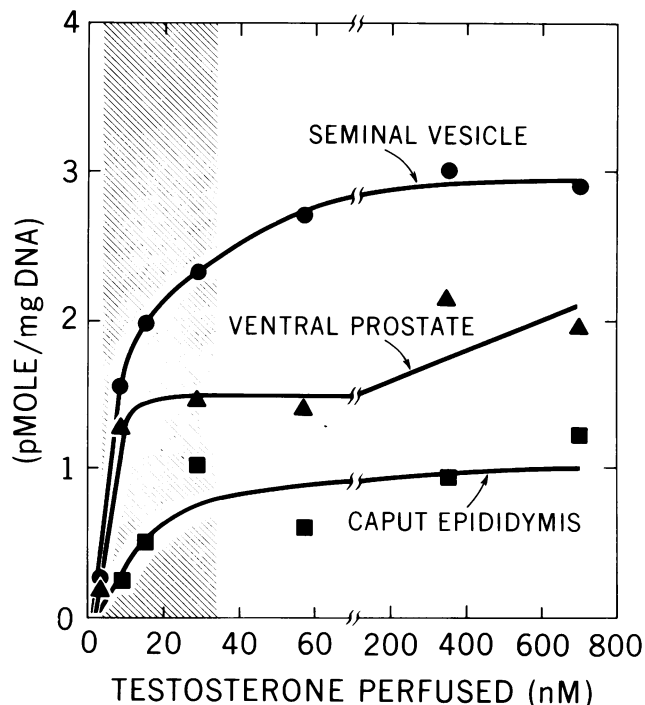


FIGURE 3. Patterns of nuclear accumulation of dihydrotestosterone in accessory sex organ nuclei from castrated rats. Hemicorpora from 24 hr castrated rats were perfused with various concentrations of testosterone. Dihydrotestosterone was extracted from the nuclei, purified by chromatography and the levels calculated. The shaded area represents the physiological range for testosterone levels in male rat blood. From Baker (4).

Mechanism of Androgen Accumulation

Evidence for specificity of dihydrotestosterone accumulation was obtained from competition experiments with testosterone and the antiandrogen, cyproterone acetate. Nuclear ^3H -dihydrotestosterone was reduced in each organ by both agents, whereas testosterone levels were reduced to a lesser extent in nuclei of the ventral prostate and seminal vesicle and not at all in epididymal nuclei. When KCl extracts of nuclei from the accessory sex organs were centrifuged on sucrose gradients, ^3H -labeled molecules sedimented in the 3S region (Fig. 4). Chromatographic separation of the androgens revealed that dihydrotestosterone was bound to the 3S molecules, whereas testosterone appeared to be unbound.

To demonstrate that apparent saturation of nuclei with dihydrotestosterone was not due to a limited amount of steroid in cytoplasm, dihydrotestosterone was measured in samples of crude cytoplasm from the hemicorpora used for the nuclear accumulation studies. Cytoplasmic dihydrotestosterone levels rose progressively over the whole

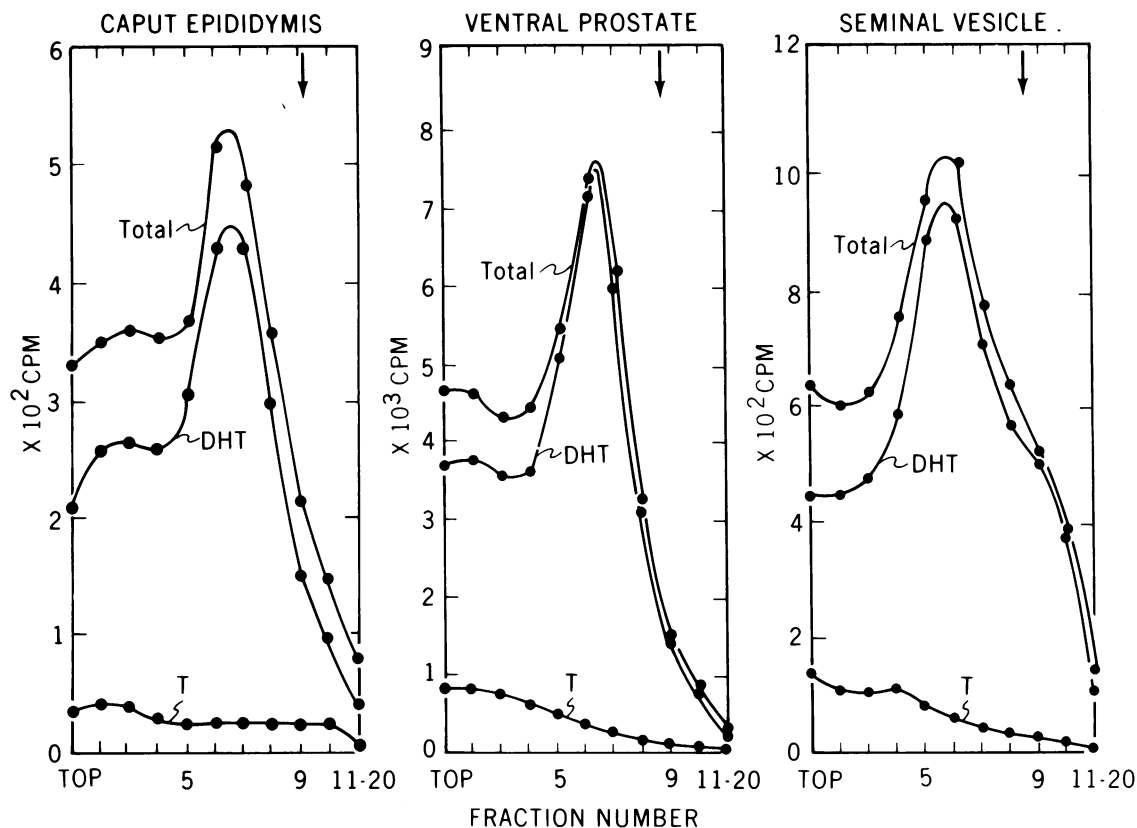


FIGURE 4. Sucrose gradient profiles of KCl extracts of accessory sex organ nuclei from 24 hr castrated hemicorpora. Two preparations were perfused with $40nM$ 3H -testosterone and the organs pooled. Arrows indicate the position of the ^{14}C -bovine serum albumin peak. From Baker (4).

range of testosterone concentrations studied. Thus, restricted formation of dihydrotestosterone in cytoplasm could not explain nuclear saturation (4).

In summary, dihydrotestosterone is the major androgen in the nuclei of accessory sex organs of castrate rats perfused with testosterone. The nuclear accumulation of dihydrotestosterone is specific, saturable and presumably dependent upon androgen receptors.

Comparison of Castrated and Testosterone-Treated Hypophysectomized Rats

In order to contrast the nuclear accumulation of androgens in the accessory sex organs and testes of the same animals, hypophysectomized rats were treated with testosterone to regenerate the accessory sex organs. In these animals, the ventral prostate and seminal vesicle weights were similar to those from 24 hr castrated preparations but the epididymides were similar to those from hypophysectomized rats. The nuclear androgen levels in accessory sex organs of testosterone-treated hypo-

physectomized and 24 hr castrated hemicorpora were measured during perfusion with $20nM$ testosterone. Dihydrotestosterone levels in epididymal and seminal vesicle nuclei were not significantly different in the two preparations. However, the hypophysectomized hemicorpora had significantly lower androgen levels in prostatic nuclei (4). Prolactin treatment increased the saturation levels of dihydrotestosterone in prostatic nuclei (10).

Comparison of Androgens in Accessory Sex Organ and Testicular Nuclei

The patterns of nuclear androgen accumulation in perfused accessory sex organs and testes from testosterone treated hypophysectomized rats are shown in Figure 5. As in the above studies of castrate rats (Fig. 3), nuclear dihydrotestosterone rose initially with increasing concentrations of perfused testosterone but then showed evidence of saturation. Although there were quantitative differences, the patterns of nuclear androgen accumulation in the accessory sex organs were similar, and the tes-

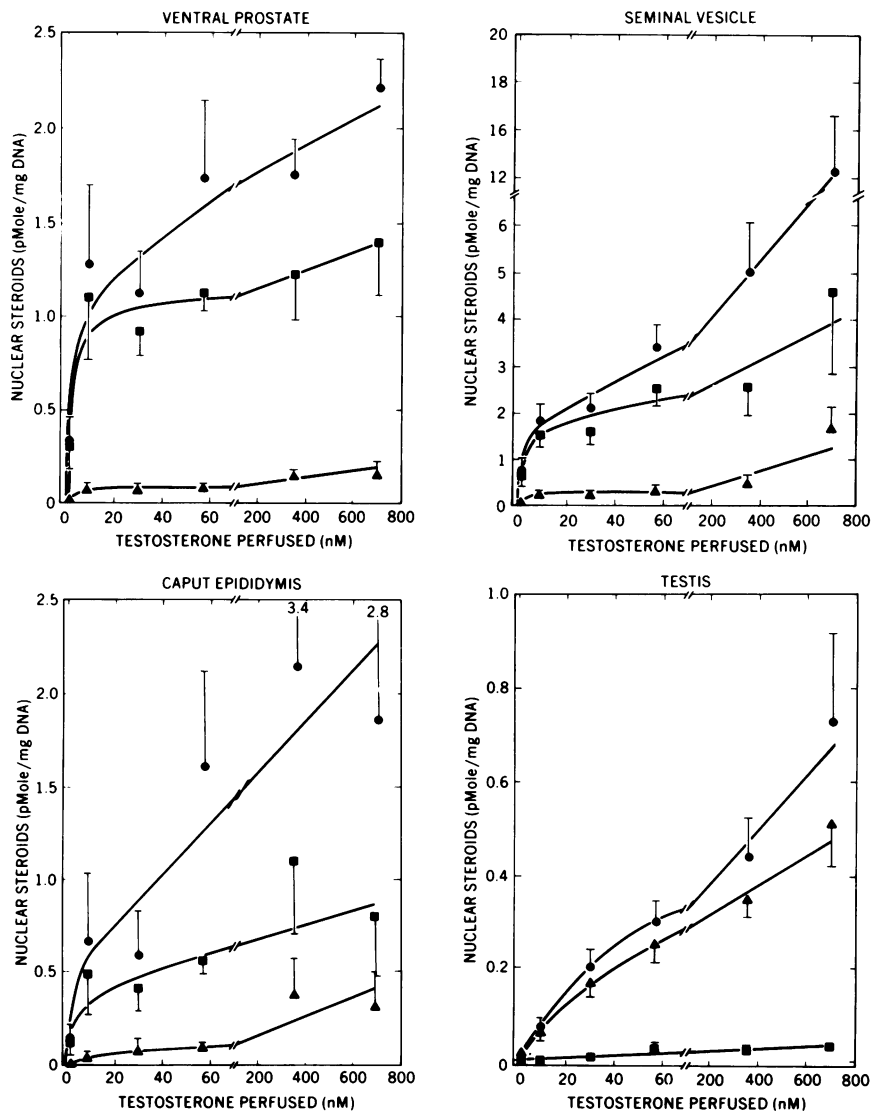


FIGURE 5. Patterns of accumulation of androgens in nuclei of accessory sex organs and testes from hypophysectomized testosterone treated rats: (●) total androgen; (■) dihydrotestosterone; (▲) testosterone. Each point and error bar represents the mean and SEM of 3-9 results. From Baker (4).

tosterone levels were lower than the dihydrotestosterone levels at each concentration of testosterone studied. In contrast, the pattern of androgen accumulation in testicular nuclei was different (Fig. 5). Firstly, testosterone was the major androgen, and very little dihydrotestosterone was detectable. Secondly, the nuclear androgen levels were much more lower in testes than in accessory sex organs. Thirdly, testosterone accumulation in testicular nuclei continued to increase over the range of initial testosterone concentrations (1–700 nM) perfused, and a plateau was not apparent.

The lack of obvious saturation of the testosterone

accumulation in testicular nuclei could be explained by the presence of relatively large amounts of nonspecifically bound testosterone. This suggestion was supported by the finding that perfusion of a 1000-fold excess of radioinert testosterone reduced the ^3H -testosterone in the nuclei by less than 50%. Similar results were obtained when 30 nM ^3H -testosterone was perfused with a 100-fold molar excess of cyproterone acetate. In addition, to nonspecific binding, subsequent studies demonstrated that cytoplasmic contamination contributed to the observed pattern of testosterone accumulation in testicular nuclei (4).

Androgen Binding in Testicular Nuclei

Further evidence that receptors were involved in the nuclear uptake of androgens in perfused testes was found in sucrose gradient profiles of KCl extracts of the nuclei. In initial experiments, large amounts of free steroid obscured a 3S peak (Fig. 6A). This was removed by charcoal treatment of the KCl extracts. In order to obtain sufficient nuclear androgen for identification by thin layer chromatography, a number of testes were perfused with the isolated organ method and the fractions from several sucrose gradients pooled. Under these conditions, testosterone was bound to 3S molecules in the nuclei of testes from mature hypophysectomized rats perfused with ^3H -testosterone (Fig. 6B).

To determine whether dihydrotestosterone could also bind in testicular nuclei, isolated testes were perfused with ^3H -dihydrotestosterone. When charcoal-adsorbed nuclear extracts were sedimented through sucrose gradients, a ^3H -dihydrotestosterone peak was present in the 3S region (Fig. 6C). These studies indicate that both testosterone and dihydrotestosterone can be bound to 3S molecules in testicular nuclei of mature hypophysectomized rats.

The above observations are in keeping with the results of others, indicating that dihydrotestosterone is the major intranuclear androgen in prostate and seminal vesicle and caput epididymis (11-13), whereas testosterone is observed in testicular nuclei. Smaller amounts of testosterone were also present and were displaceable

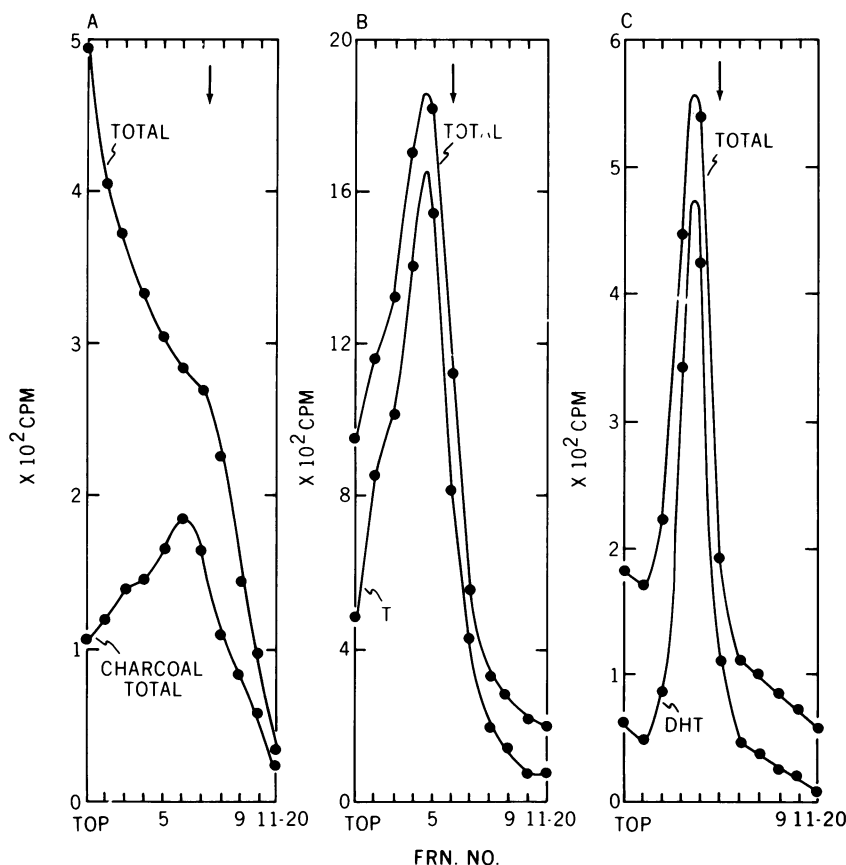


FIGURE 6. Sucrose gradient profiles of KCl extracts from perfused testes of 21-day hypophysectomized rats (4). (A) Effect of charcoal adsorption of the KCl extract. Seven testes were perfused with 85mM ^3H -testosterone. The testes were pooled and the nuclei isolated and extracted with 0.4M KCl. A sample (0.35 ml) of the extract was layered on one gradient (total) and another sample was layered on a second gradient after adsorption with dextran-coated charcoal (charcoal total). Total tritium was measured in the gradient fractions. The positions of the ^{14}C -bovine serum albumin marker are indicated by arrows. (B). Testosterone perfusion. Six testes were perfused with 85mM ^3H -testosterone and the KCl extract of the nuclei placed on the sucrose gradients after charcoal adsorption. Total tritium (total) and ^3H -testosterone (T) purified by thin layer chromatography, were measured in pooled fractions from five gradients. There was very little ^3H -dihydrotestosterone ($<50 \text{ cpm/fraction}$). (C) Dihydrotestosterone perfusion. Four testes were perfused with 167nM ^3H -dihydrotestosterone and the KCl extract of the nuclei placed on gradients after charcoal adsorption. Total tritium (total) and ^3H -dihydrotestosterone (DHT) were measured in pooled fractions from three gradients. From Baker (4).

from prostate and seminal vesicle nuclei, suggesting that testosterone was specifically bound to intranuclear receptors. However, after centrifugation of nuclear extracts on sucrose gradients, only dihydrotestosterone was bound to 3S macromolecules. Similar observations of the behavior of androgen receptor complexes on sucrose gradients led several investigators to suggest that prostatic receptor which enters the nucleus is highly specific for dihydrotestosterone (14, 15). The apparent inconsistency of these findings was partially explained when it was noted that testosterone dissociated from the prostatic cytoplasmic receptor during centrifugation on sucrose gradients. The present results suggest that under physiologic conditions, 10–20% of the specifically bound androgen in prostate and seminal vesicle in nuclei is testosterone, even though this cannot be demonstrated on gradients. The inconsistency in these observations still remains to be explained, in view of the fact that testosterone does not dissociate from the androgen receptor of rat testis and mouse kidney during centrifugation.

Testosterone versus Dihydrotestosterone in Testicular Nuclei: Influence of Age and Hormone Treatments

As both androgens could bind in testicular nuclei, the possibility that the proportion of testosterone and dihydrotestosterone would be related to testicular 5 α -reductase activity was examined. Previous studies showed that testicular 5 α -reductase activity was low in rats greater than 60 days of age (16) and was increased by estrogen treatment (17), whereas it was high in 40-day-old rats (16) and reduced by testosterone treatment (18). Thus, preparations from these four groups were perfused with ³H-testosterone. Since the nuclei androgen levels could not be determined precisely because of the presence of variable amounts of endogenous testosterone in the four groups, the relative amounts of testosterone and dihydrotestosterone were compared (Fig. 7). The mature rats had a low dihydrotestosterone:testosterone ratio which was increased by estrogen treatment. The high ratio observed in 40-day-old animals was reduced by testosterone treatment. Thus, a dihydrotestosterone content of testicular nuclei was altered by age and treatments known to change testicular 5 α -reductase activity, suggesting that dihydrotestosterone accumulation correlates with the activity of this enzyme.

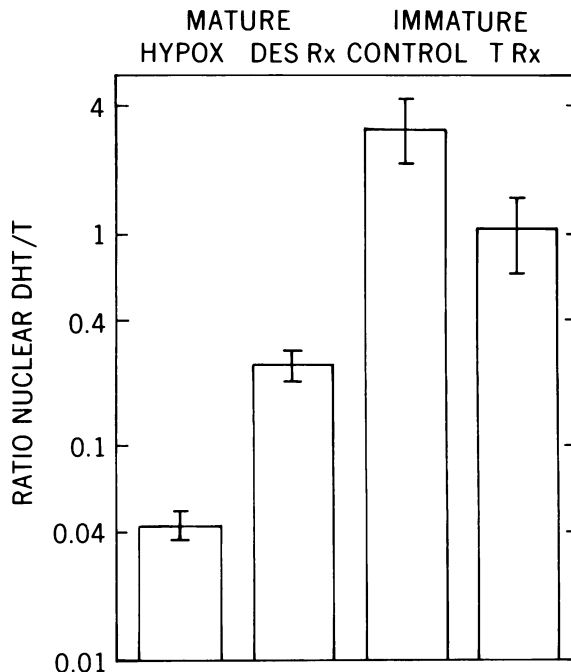


FIGURE 7. Effect of age and hormone treatment on androgen accumulation in testicular nuclei. The ratio of dihydrotestosterone to testosterone (mean \pm SEM) in ethanol extracts of testicular nuclei from hemicorpora perfused by the standard method with ³H-testosterone was calculated for: 37 rats hypophysectomized at age 60 days of age and used 21–35 days later (mature, hypox); six 108-day-old rats treated with diethylstilbestrol (mature, DESRx); four 40-day-old rats (immature, control); and, four 40-day-old rats treated with testosterone (immature, TRx). From Baker (4).

Conclusion

The feasibility of testicular perfusion was established by development and characterization of methods for humans (19), bulls (20), horses (21), dogs (22, 23), rabbits (24, 25), and rams (26). However, these procedures require considerable technical expertise to cannulate the internal spermatic artery in order to establish adequate perfusion without a long period of ischemia. Although a method for short-term perfusion of rat testis has been reported (27), it was not evaluated critically. Subsequent experiments from our laboratory have established two techniques: selective and isolated testicular perfusion (3). Of these two procedures, the isolated method was superior when only testes were to be studied. However, the hemicorpus preparation offers the advantage of studying testes along with the remainder of the reproductive tract. The advantages and disadvantages of these techniques have been thoroughly described (1–4, 10). Observations suggest that these procedures will be useful for study of drug as well as hormone metabolism and mechanism of action.

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